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Central Ghrelin Reduce Water Intake in Japanese Quail

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Abstract: **This study was conducted to investigate whether ghrelin has an anti-dipsogenic effect as seen in the neonatal chicks and eel, when administered centrally in Japanese quail Coturnix coturnix japonica). Intracerebroventricular (ICV) administration of avian ghrelin (Chicken ghrelin) reduced water intake in Japanese quail under both *ad libitum* and 16-h water deprived drinking conditions at doses ranging from 0.5 to 1.0 nmol/quail. This inhibitory effect was also observed when 0.5 nmol of rat ghrelin was centrally administered. On the other hand, 0.5 nmol des-acyl rat ghrelin did not reduce water intake. To elucidate the mechanism underlying the effect of ghrelin on water intake, avian B-type (or brain) natriuretic peptide (BNP), an anti-dipsogenic peptide in mammals, was centrally administered at doses ranging from 0.1 to 1.0 nmol/quail. BNP did not affect water intake in quail under both normal and water-deprived drinking conditions. These findings might suggest that ghrelin acts as an anti-dipsogenic peptide through the GHS receptor in the Japanese quail. This finding might reveals a new role for ghrelin in body fluid homeostasis.**

Keywords: **Japanese quail, BNP, Ghrelin, ICV administration, Water intake.**

1. INTRODUCTION

Ghrelin, a novel 28-amino acid peptide, was originally isolated from the stomachs of rats, mice and humans as an endogenous ligand for the growth hormone (GH) secretagogue receptor (GHS-R) (Kojima et al., 1999; Cowley et al., 2003). It has a unique structure, containing a Ser3 residue that is modified by *n*-octanoic acid. This octanoyl modification is essential for receptor binding and the subsequent expression of biological activity (Kojima et al., 1999). Exogenous ghrelin elicits various physiological functions in the brain such as feeding control, growth hormone (GH) release, and gastric acid secretion (Kojima & Kangawa 2005). Effects of ghrelin on feeding behavior have been well examined in mammals (Horvath et al., 2001), and it is thought that endogenous ghrelin acts physiologically to increase feeding behavior in mammals (Nakazato et al., 2001). It is known that the orexigenic effects of central ghrelin are mediated by neuropeptides such as neuropeptideY(NPY), agouti-related protein (AgRP) and orexin (Nakazato et al., 2001; Toshinai et al., 2003).

In avian species, ghrelin has been purified and identified in various tissues (such as the proventriculus, brain, intestine, lung and spleen) in six avian species (chickens, Japanese quail, duck, geese, Emu and turkey) (Kaiya et al., 2002; 2007; 2008; 2009; 2013a; 2013b; Mark et al., 2010). Ghrelin was determined to be present in various non-mammalian vertebrates, and its physiological effects were gradually revealed in chickens (Kaiya et al., 2002), Japanese quails and other avian species (Kaiya et al., 2007; 2008; 2009; 2011, 2013a; 2013b). This form is composed of 26 amino acids, has an octanoylated Ser3 and shows 54% total sequence identity and 100% N-terminal-region identity [Gly1-Pro7] with rat and human ghrelin (Kaiya et al., 2002). So chicken and japanese quail ghrelin shows 85 % total sequence identity and 100% N-terminal-region identity [Gly1-Pro7] with rat and human ghrelin (Kaiya et al., 2002; 2008; 2011). The GHS-R is found in the pituitary, brain, liver, intestine, and spleen (Geelissen et al., 2003; Tanaka et al., 2003). In addition, two isoforms of the chicken GHS-R (cGHS-R1a and cGHS-R1aV) have been generated by alternative splicing of a primary

transcript. cGHS-R1a shows strong amino acid sequence identity (68%) with the corresponding parts of the mammalian GHS-R1a cDNA product, while cGHS-R1aV lacks the transmembrane-6 domain due to a 48-bp deletion (Tanaka et al., 2003). Moreover, Several types of GHS-R, namely GHS-R1a-L, GHS-R1a-S, GHS-R1aV, GHS-R1b, GHS-R1bV and GHS-R1tv-like receptor, were identified in Japanese quail and the amino acid sequence of quail GHS-R1a-L was 98% identical to that of chicken GHS-R1a (Kitazawa et al., 2009). The region-specific expression pattern in Japanese quail was almost the same as that in the chicken and the regions in which ghrelin acts were consistent with the distribution of GHS-R1a mRNA (Kitazawa et al., 2009).

As seen in mammals, ghrelin increases the release of GH after intravenous administration in the chicken (Baudet & Harvey 2003; Kaiya et al., 2002). ICV injection of ghrelin or a GHS-R agonist, GHRP-2, strongly inhibits food intake in chicks (Furuse et al., 2001); an effect opposite that seen in rats (Nakazato et al., 2001). The anorexic effect has also been observed in another avian species, quail (Shousha et al., 2005), suggesting that the action of ghrelin on food intake is likely to be unique to birds.

It is reported that intracranial administration of ghrelin inhibited water drinking in seawater acclimated eels (Kozaka et al., 2003) and neonatal chicks [Tachibana et al., 2006]. This suggests the possibility that ghrelin, administered into the brain, may affect water intake in Japanese quail. The purpose of the present study was to investigate whether ICV administration of ghrelin affects water intake in Japanese quail. In addition, natriuretic peptide (NP) is known to be a potent anti-dipsogenic factor in mammals (Katsuura et al., 1986; Itoh et al., 1988; Tarjan et a., 1988; Fregoneze et al., 1989; Uehara et al., 1990; Weisinger et al., 1992). We examined the effect of chicken B-type NP (BNP), originally isolated from chicken heart (Miyata et al., 1988), on water intake in japanese quail, to investigate the interaction of BNP and the effect of ghrelin. We decided to use adult Japanese quails. This was mainly because the growth curve of young birds is steep, so food intake and consequently water intake vary widely from day to day, whereas adult birds have ceased growing, and therefore body weight gains, food intake and water intake are not subject to such variability. Furthermore, it is technically easier to implant indwelling icv cannulae into adult birds. Also, we used adult male quails to avoid the effect of female reproductive hormones on water intake and their interference with the injected peptides on water intake or other parameters.

2. MATERIALS AND METHODS

Animals:

Adult male Japanese quail (*Coturnix coturnix japonica*) were housed in individual net cages (W: 14 x L: 26 x H: 17 cm) in a room with continuous lighting and a temperature of 28±1 ºC, and were given free access to food and water. All procedures were performed in accordance with the Japanese Physiological Society's guidelines for animal care.

Surgical procedures:

For implantation of the icv cannula, each bird was anesthetized with 5% sodium pentobarbital (1.4 µl/g body weight) and placed in a stereotaxic frame. A stainless steel guide cannula (outer diameter 550 µm, length 14 mm) was stereotaxically implanted into the third cerebral ventricle using a modification of a previously reported method (Bayle et al., 1974). The coordinates were 5mm anterior to the interaural axis and 6.5 mm below the dura at the midline. One stainless steel anchoring screw was fixed to the skull, and the guide cannula was secured in place with acrylic dental cement. The birds were returned to their individual cages and allowed to recover for at least 4 days. They were acclimatized to handling every day before the start of the experiments.

Synthetic octanoylated chicken ghrelin-26 (chicken ghrelin), octanoylated rat ghrelin (rat ghrelin), des-acyl rat ghrelin (desacylghrelin) and chicken BNP were dissolved in a 5% mannitol solution, to prevent the absorption of peptides to the wall of tubes or syringes, with 0.1% Evans Blue. The control group was administered with the mannitol solution. The injected volume was 10 µl in all experiments. The icv injections were administered through the implanted guide cannulae without anesthesia or restraining of the birds (Davis et al., 1979). Briefly, the head of the bird was held with an acrylic device, which positioned a hole in a plate overlying the skull immediately over the third cerebral ventricle. Then a microsyringe was inserted into the third cerebral ventricle through the hole and the test solution was administered. This injection method is considered to not induce stress in the birds since the ICV injection of 5% mannitol solution, which was used for control injections in the present study, does not alter plasma corticosterone (an indicator for stress response)

when compared with intact birds (Saito et al., 2005). At the end of experiment, birds were sacrificed with an intraperitoneal overdose of sodium pentobarbital.

Prior to administration, the birds were weighed and assigned to an experimental groups based on their body weight. The average body weight (110–120 g) in each group was kept as uniform as possible. Water intake was determined at 30, 60, 120 and 180 min after peptide administration by measuring the disappearance of water from the pre-weighed water cup with a digital balance with a precision of 0.001 g. Furthermore, evaporation of water during experiment was also measured, and the data was used for correction of water intake. In addition, we have uniformly distributed control and experimental groups at the experimental room as the error becomes to be minimum. Food was freely available until the administration, but was removed thereafter.

Verification of cannula placement:

At the end of the experiments, proper placement of the cannulae was verified by administering Evans Blue dye (10 µl), followed by sacrifice and brain sectioning (20 µm intervals). Data for birds lacking dye in the third ventricle were excluded from the analysis.

Effect of chicken ghrelin on water intake:

Birds were administered with saline (control), 0.05, 0.5 or 1.0 nmol chicken ghrelin under *ad libitum* and 16-h water-deprived drinking conditions.

Effect of rat ghrelin and des-acyl ghrelin on water intake:

Birds were administered with saline (control), 1.0 nmol rat ghrelin or 1.0 nmol des-acyl ghrelin after a 16-h water-deprived drinking conditions.

Effect of chicken BNP on water intake:

Birds were administered with saline (control), 0.1, 0.5 or 1.0 nmol chicken BNP under *ad libitum* and 16-h water-deprived drinking conditions.

Statistical analysis:

Data were analyzed by analysis of variance (ANOVA) and post hoc Fisher's test, and the results are expressed as means ± SEM. A P-value less than 0.05 is considered to be significant.

3. RESULTS

Fig. 1 shows the effect of chicken ghrelin on water intake under *adlibitum* drinking conditions. Ghrelin significantly ($P < 0.01$) inhibited water intake. The inhibitory effect showed a time-dependent interaction. A significant decrease in water intake was observed at 60, 120 and 180 min after the administration of 0.5 and 1.0 nmol chicken ghrelin.

Fig. 2 shows the effect of chicken ghrelin in 16-h water-deprived conditions. The inhibitory effect was significant ($P < 0.05$). Although 0.05 nmol ghrelin did not affect water intake at any time point, higher doses (0.5 and 1.0 nmol) of ghrelin significantly inhibited water intake at 60 min after administration. Water intake was still inhibited at 120 and 180 min after administration of the highest dose (1.0 nmol) of chicken ghrelin.

Fig. 3 shows the changes in water intake after administration of rat ghrelin and des-acyl ghrelin. Rat ghrelin significantly ($P < 0.05$) inhibited water intake at 60 and 120 min after the administration. In contrast, des-acyl ghrelin did not affect water intake at any time point.

Fig. 4 Shows the effects of chicken BNP on water intake under *adlibitum* drinking conditions. Chicken BNP ranging from 0.1 to 1.0 nmol did not affect water intake under *ad libitum* drinking condition.

Fig. 5 Shows the effects of chicken BNP on water intake under 16-h water-deprived drinking conditions. Chicken BNP ranging from 0.1 to 1.0 nmol did not affect water intake under 16-h water-deprived drinking conditions.

4. DISCUSSION

There are few reports about the effect of ghrelin on water intake in either mammalian or non-mammalian species. This is the second report to demonstrate an inhibitory effect of ghrelin on water intake in avian species and our result is similar to that seen in eels (Kozaka et al., 2003) and neonatal chicks (Tachibana et al., 2006). Here, we examined the effect of central ghrelin on water intake in Japanese quails. The ICV administration of ghrelin inhibited water intake under both *ad libitum* drinking and 16-h water-deprived conditions. The administered ghrelin was effective at dose more than 0.05 nmol. This dose is comparable to that which inhibits food intake in Japanese quail (Shousha et al., 2005). This indicates that central ghrelin acts not only as a feeding-inhibitory peptide, but also as an anti-dipsogenic peptide in Japanese quail and would induce both effects at the same time.

The effect of ghrelin on water intake were not observed during the first 30 min after administration but were evident at 60, 120 and 180 min after administration under *ad libitum* and water-deprived conditions, respectively. Under an *ad libitum* drinking condition, birds do not drink water if food is not available. The quantity of water intake at 30 min after the administration may be too small to detect. Water intake decreased by ghrelin under an *ad libitum* drinking condition. In contrast, dehydrated birds drank much water after restricted water was replaced. Thus, ghrelin could not inhibit the strong motivation to drink during the first 30 min. However, there was a tendency to reduce water intake at 30 min after a ghrelin administration even under dehydration condition, although it is not statistically significant. This suggests the potent anti-dipsogenic effect of ghrelin in Japanese quail.

Regarding to the effect of rat acyl and des-acyl ghrelin on water intake in Japanese quail, our results revealed that des-acyl ghrelin had no effect on water intake. In contrast, both avian ghrelin (chicken ghrelin) and rat ghrelin inhibited water intake in Japanese quails. The N-terminal seven amino acids of ghrelin are identical between chicken and rat (Kaiya et al., 2002). In fact, all avian ghrelin peptides display total conservation of the first seven N-terminal amino acids. Moreover, all avian mature ghrelin peptides are predicted to be 26 amino acids in length with the exception of turkey ghrelin (Richards & McMurtry 2010). The N-terminal tetrapeptides including octanoylation is known to be the "active core" of ghrelin to bind to the GHS-R and to elicit biological actions (Bednarek et al., 2000; Matsumoto et al., 2001). Des-acyl ghrelin does not induce intracellular Ca^{2+} influx in cells expressing GHS-R 1a (Kojima et al., 1999; Matsumoto et al., 2001). Our results indicate that the conserved N-terminal sequence and octanoylation of avian and rat ghrelin are essential for this anti-dipsogenic effect through GHS-R1a, and that the observed response is the specific effect of ghrelin.

The mechanism of inhibitory effect of ghrelin on water intake is still unclear because few reports (one in eel (Kozaka et al., 2003), one in neonatal chicks (Tachibana et al., 2006) and one in rats (Hashimoto et al., 2007) on this phenomenon are available. It is possible that the anti-dipsogenic effect of ghrelin is mediated by some neuronal factors. Neuropeptides and monoamines are possible mediators of this effect. However, possible involvement of monoamines would be excluded because it has reported that ICV ghrelin does not change any monoamine levels in the chick brain (Saito et al., 2002). Next, it is reported that the anorexic effect of ICV ghrelin in Japanese quail (Shousha et al., 2005) and chicks (Furuse et al., 2001; Saito et al., 2002), and recently it was revealed that corticotrophin releasing factor (CRF) system mediates the effect (Saito et al., 2005). In preliminary experiments we found that ICV ghrelin inhibits both food and water intake (unpublished data). In addition, administered doses in this study were comparable to that induced inhibition of food intake in Japanese quail (Shousha et al., 2005). These results suggest a possibility that CRF might mediate the central effect of ghrelin on water intake. However, it has been reported that ICV administration of CRF does not inhibit water intake in avian species such as seen in chickens (Denbow et al., 1999; Meade et al., 2003) indicating that CRF is not involved in this anti-dipsogenic effect of ghrelin.

It has been reported that atrial NP (ANP) induced a potent anti-dipsogenic peptide in various vertebrates such as rats (Katsuura et al., 1986), rabbits (Tarjan et al., 1988), sheep (Weisinger et al., 1992) and eels (Kozaka et al., 2003; Tsukada et al., 2005). In contrast, it has been reported that human ANP induces copious drinking when administered ICV in Japanese quail (Okawara et al., 1986). We therefore can hypothesize that NP acts as a candidate mediator of the effect of ghrelin. In avian species, NP was isolated from the heart of chicken and Japanese quail and designated as chicken alpha-atrial NP (α - ANP) (Miyata et al., 1988; Mifune et al., 1996). However, the structure is not similar to ANP but instead belongs to the BNP lineage (Inoue et al., 2005). BNP also acts as an anti-dipsogen in rats when administered ICV (Itoh et al., 1988; Fregoneze et al., 1989; Uehara et al., 1990). However, it is reported that ICV of BNP in chickens inhibited water intake but there is no report about the effect of ICV of BNP on drinking in Japanese quail. In mammals, ANP at a

dose of less than 1 nmol, and BNP at doses from 1.5 to 2 nmol show an anti-dipsogenic effect. Therefore, we examined chicken BNP ranging from 0.1 to 1.0 nmol with taking mammalian NP studies into consideration. No significant effect on water intake was observed following the BNP administration. This is the first report about the effect of BNP on water intake in Japanese quail but there is an earlier report about the effect of BNP on water intake in neonatal chicks (Tachibana et al., 2006). Ghrelin was effective at doses of 0.5 and 1.0 nmol in this study. In contrast, higher dose of chicken BNP did not show any effect either in neonatal chicks (Tachibana et al., 2006) or in this study in Japanese quail. However, considering that ANP exhibits a dipsogenic effect in the Japanese quails (Okawara et al., 1986), it is unlikely that BNP mediated an anti-dipsogenic effect by ghrelin in quail brain. Other neuropeptides such as substance P and leu^5-enkephalin exhibit an inhibitory effect on drinking in Japanese quail (Uemura et al., 1984; 1985). There is no information about interaction of ghrelin among these neuropeptides in avian brain.

The results of the earlier study of Tachibana et al. 2006 in chicks and the results of our study in Japanese quail demonstrated an inhibitory effect of central ghrelin on water drinking under ad libitum or restricted conditions. These results suggest that ghrelin acts on brain areas other than the feeding center. The possible areas for the regulation of water intake are equivalent to the subfornical organ, preoptic area, organum vasculosum laminae terminalis, and area postrema in mammals. A similar inhibitory effect on water drinking has also been well documented in rats (Hashimoto et al., 2007; 2010). Interestingly, water drinking was inhibited, whereas food intake was stimulated, after ICV administration of ghrelin in rats. Normally, increased food intake is accompanied by increased drinking (prandial drinking); however, the inhibitory effect of ghrelin on water drinking appears to also inhibit prandial drinking. It is likely therefore that ghrelin targets different parts of the brain. In birds, however, ghrelin inhibits both food intake (shousha et al., 2005) and water drinking (Tachibana et al., 2006) and in this study. Denbow et al. (1999) found that decreased water intake induced by ICV administration of CRFs is a secondary response to the suppression of food intake, meaning that CRFs directly inhibit prandial drinking in chickens. Ghrelin also inhibits food intake by mediating CRFs, and possibly urocortin, as described earlier (Saito et al., 2005; Khan et al., 2008), suggesting that urocortin, which is a secondary mediator of the action of ghrelin, directly inhibits prandial drinking. Alternatively, there is the possibility that inhibition of water drinking by ghrelin may be mediated through CRFs. Different neural networks for regulating food intake and water drinking between rodents and birds could result in these distinct effects (Kaiya et al., 2013b). Further study is necessary to clarify the mechanisms of ghrelin-induced anti-dipsogenic effect in Japanese quail.

Earlier studies that reported the inhibitory effect of ghrelin on water intake in domestic chickens used the lateral ventricle as the route for central injections (Tachibana et al., 2006) whereas in our study we used the third ventricle. We also observed an inhibitory effect of ghrelin on water intake. Therefore, although the route of administration was different from that used in the previous studies in chickens, the results for both chickens and Japanese quails were the same, and both were the same to those reported for mammals (Hashimoto et al., 2007), suggesting that the site of injection does not influence the differences between birds and mammals.

From this study, we can conclude that these findings might suggest that ghrelin acts as an antidipsogenic peptide in the brain of Japanese quail. This might suggests a possible involvement of ghrelin in the regulation of body fluid homeostasis in avian species

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APPENDIX - A

FIGURE LEGENDS:

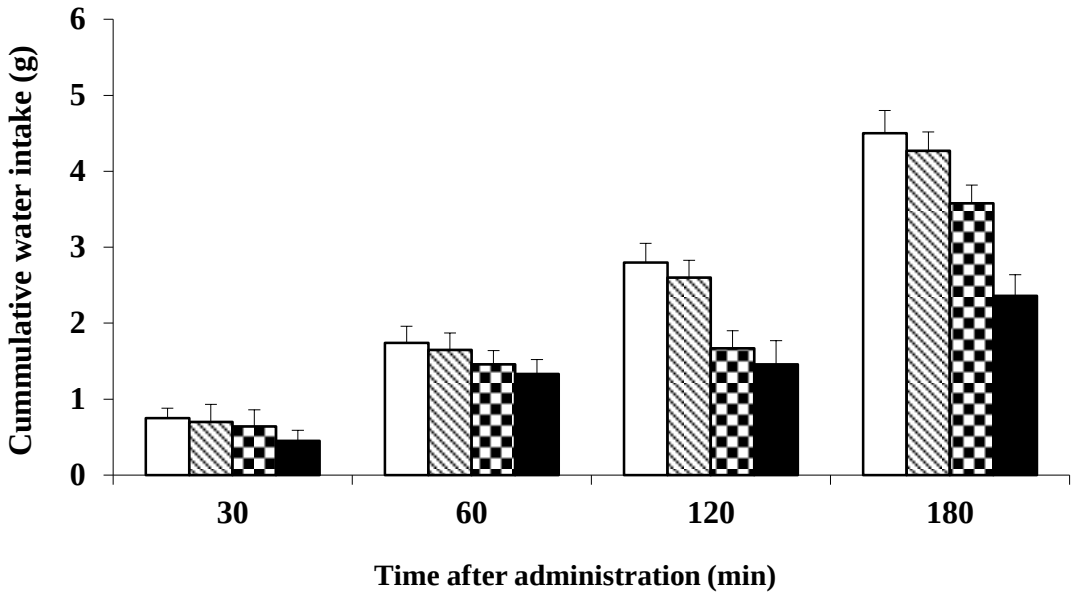

Fig. 1 Effect of ICV administration of chicken ghrelin (saline (control); 0.05 nmol; 0.5 nmol; 1.0 nmol) on water intake under *ad libitum* drinking conditions. Data are expressed as mean ± SEM (n = 8). *Significantly different from the control group ($P < 0.05$) at each time point.

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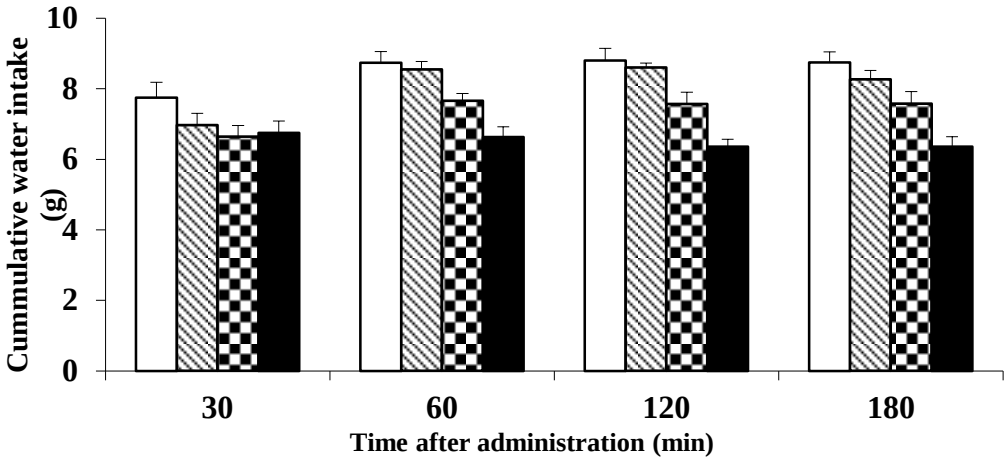

Fig. 2 Effect of ICV administration of chicken ghrelin (saline (control); 0.05 nmol; 0.5 nmol; 1.0 nmol) on water intake under 16-h water-deprived drinking conditions. Data are expressed as mean ± SEM (n = 8). *Significantly different from the control group ($P < 0.05$) at each time point.

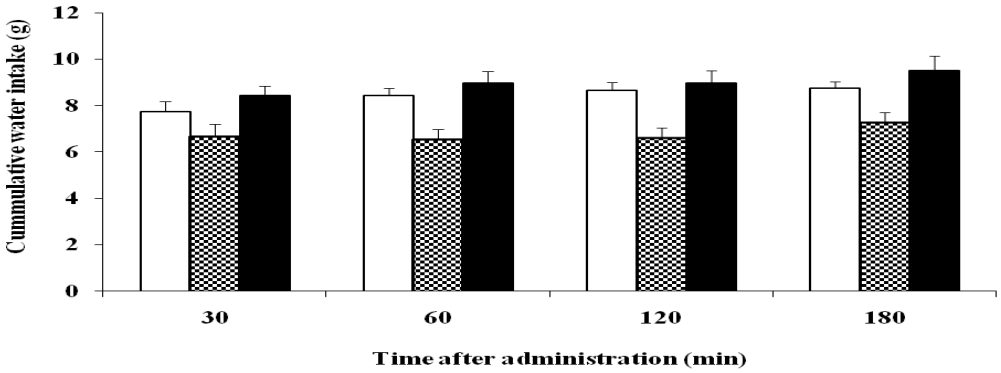

Fig. 3 Effect of ICV administration of rat ghrelin or rat des-acyl ghrelin on water intake under 16-h water-deprived drinking condition. The doses administered were: saline (control); 1.0 nmol rat ghrelin; 1.0 nmol rat des-acyl ghrelin. Data are expressed as mean ± SEM (n = 8). *Significantly different from the control group ($P < 0.05$) at each time point.

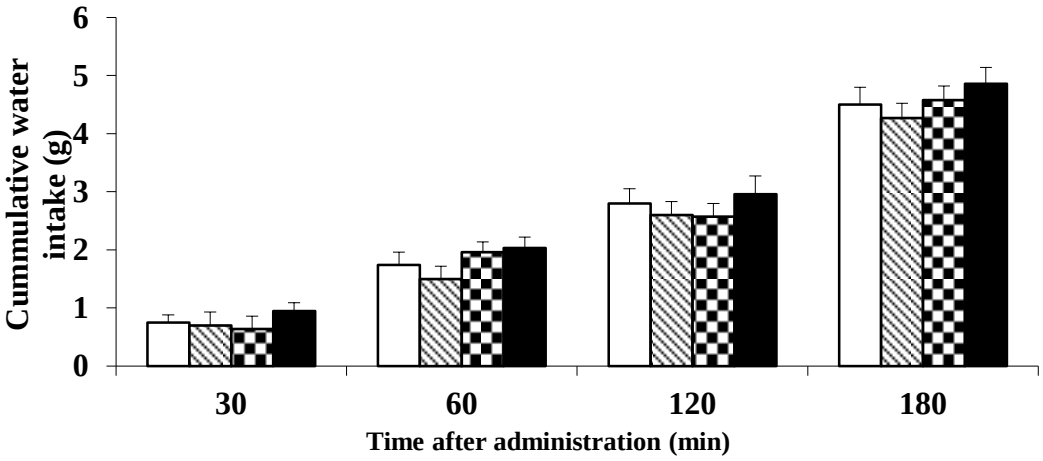

Fig. 4 Effect of ICV administration of chicken BNP (saline (control); 0.1 nmol; 0.5 nmol; 1.0 nmol) on water intake under *ad libitum* drinking conditions. Data are expressed as mean ± SEM (n = 8). No effect on water intake was seen by peptide administration.

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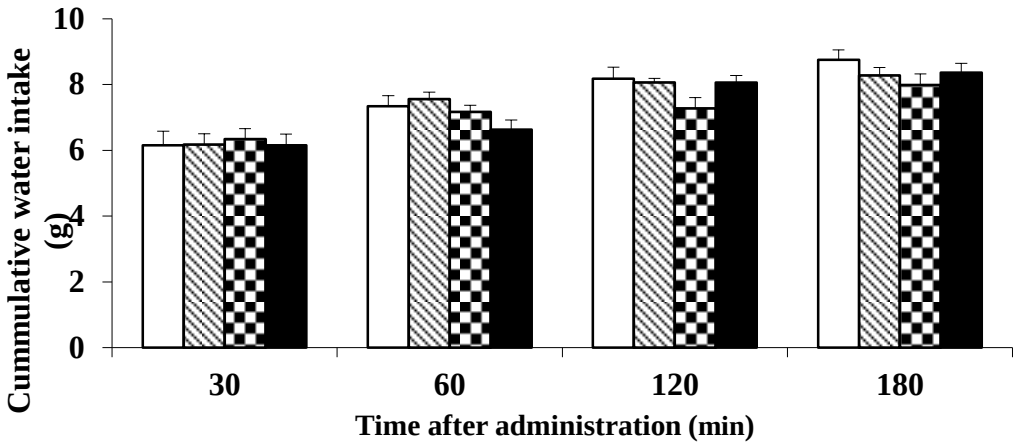

Fig. 5 Effect of ICV administration of chicken BNP (saline (control); 0.1 nmol; 0.5 nmol; 1.0 nmol) on water intake under 16-h water-deprived drinking conditions. Data are expressed as mean ± SEM (n = 8). No effect on water intake was seen by peptide administration.